

Astrocytic calcium/zinc binding protein S100A6 over expression in Alzheimer's disease and in PS1/APP transgenic mice models

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Abstract

Astrocytes recruitment and activation are a hallmark of many neurodegenerative diseases including Alzheimer's disease (AD). We have previously observed an overexpression for S100A6 protein, a $\text{Ca}^{2+}/\text{Zn}^{2+}$ binding protein presenting more affinity for zinc than for calcium, in amyotrophic lateral sclerosis (ALS). Here we demonstrated in AD patients but also in two different AD mouse models, that astrocytic S100A6 protein was homogeneously up-regulated within the white matter. However, within the grey matter, almost all S100A6 immunoreactivity was concentrated in astrocytes surrounding the $\text{A}\beta$ amyloid deposits of senile plaques. These S100A6 neocortex labelled astrocytes were also positive for the glial fibrillary acidic protein (GFAP) and S100B protein. Contrasting with S100A6, the distribution for S100B and GFA astrocytic labelled cells was not restricted to the $\text{A}\beta$ amyloid deposit in grey matter, but widely distributed throughout the neocortex. Coupling the knowledge that biometals such as zinc are highly concentrated in the amyloid deposits in AD and S100A6 having a high affinity for Zn^{2+} may suggest that S100A6 plays a role in AD neuropathology.

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1. Introduction

S100A6, previously called calcyclin, is a small EF-hand acidic $\text{Ca}^{2+}/\text{Zn}^{2+}$ binding proteins of ~11 kDa belonging to the large S100 protein family. This latter comprise, to date, 23 members, of which 16 genes are clustered on human chromosome 1q21 [1]. In neoplasia, frequent chromosomal rearrangements within 1q21 occur and deregulate S100 genes expression giving a modified pattern within neoplastic tissues [2]. Therefore, S100 Ca^{2+} -binding proteins became useful tools for pathologists, both as diagnostic and predictive markers. Likewise, S100A6 expression appeared to be induced or up-regulated in several tumour cell lines

presenting high metastatic activity [3,4]. S100A6 is expressed in a cell- and tissue-specific manner and preferentially in the G1 phase of the cell cycle, during development and after birth [5,6]. S100A6 is also able to control intracellular functions such as protein phosphorylation regulation [7], enzyme activities [8], Ca^{2+} homeostasis and microtubule dynamics [9]. S100A6 has been studied in rat brain, and its expression is restricted to neurons of some limbic system nuclei and in a few subpopulations of astrocytes in the white matter (e.g. the corpus callosum, cingulum, external capsule) [10]. In normal aged patients, a very weak neocortical up-regulation of S100A6 in astrocytes has been reported [6]. In amyotrophic lateral sclerosis (ALS), S100A6 is overexpressed within astrocytes associated to the neurodegenerative lesions [11,12]. Here, we have studied the S100A6 immunoreactivity in Alzheimer's

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disease (AD) the most common type of dementia in the elderly population. AD is neuropathologically characterized by the presence of neurofibrillary tangles (NFT) [13], senile plaques (containing extracellular deposits of A β amyloid) and a strong astroglial reaction. The late-onset sporadic form represents the vast majority of the cases of AD with occurrence of rare early-onset forms of familial Alzheimer's disease most often caused by mutations in three different genes encoding for the amyloid peptide precursor (APP), and presenilin 1 and 2 (PS1, PS2) proteins. Transgenic mice expressing one or several of these mutated human proteins involved in AD have proven valuable models for the study of the amyloid pathology in AD and in our study, two of these models were used in parallel with post-mortem Alzheimer human brain tissues. In AD, astrogliosis could be demonstrated by expression of astrocytic glial fibrillary acidic protein (GFAP) and S100B protein. Generally, astrogliosis appears widely distributed throughout the entire cortex and is not restricted to the vicinity of senile plaques. Here we demonstrate, through the use of S100A6 immunohistochemistry, both in AD and in the AD transgenic animals models, a particular S100A6-positive phenotype in astrocytes specifically located around the amyloid plaques deposits within the grey matter, whereas in the white matter, the S100A6 immunoreactivity appears homogeneously distributed.

2. Materials and methods

2.1. Human brain tissue preparation

Human brain samples from five clinically demented and neuropathologically confirmed cases with sporadic AD and from four neurologically healthy subjects (Table 1) were obtained from autopsies, after familial authorisation and in

agreement with the rules of the local Ethical Committee. Tissue blocks of the human temporal gyrus (T5) including hippocampus were fixed with 10% (v/v) formalin, dehydrated, embedded in paraffin and cut in tissue sections of 7- μ m-thickness.

2.2. Transgenic mice

Single transgenic APP (V747I) London mutant mice [14] and double transgenic mice for human FAD mutant PS1M146L and APP751 SL mutant (Swedish (K670N, M671L) and London (V717I) FAD mutations) were generated by crossbreeding of mice homozygote for human mutant PS1M146L with heterozygote Thy1-APP751SL mice (C57/C6), and have been previously described [15,16]. Brains from control and single and double transgenic mice aged from 2.5 to 10 months were dissected and immersion-fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin.

2.3. Antibodies

The rabbit S100B polyclonal antibody and the mouse monoclonal antibodies to GFAP and to human A β amyloid were purchased from Sigma (Belgium). The rabbit polyclonal anti-tau antibody was raised against bovine tau proteins, reacts with human and rodent tau proteins and has been described [17]. The antibody specificity for the goat anti-S100A6 has been extensively described by different groups and by us in previous studies [12] and specifically recognises the S100A6 protein in different cell types, including rat cortical neurons [10,18,19].

2.4. Immunohistochemistry

2.4.1. Single immunohistochemical labelling

Rehydrated tissue sections were treated with H₂O₂ 0.3% (w/v) in CH₃OH to inhibit endogenous peroxidase. Afterwards, sections were incubated for 1 h at room temperature with the blocking solution (5% (v/v) normal serum from swine, goat, or sheep (depending on the origin of the secondary antibody), diluted in TBS (0.01 M Tris, 0.15 M NaCl, pH 7.4)). S100A6 immunohistochemical labelling was performed using the avidin–biotin peroxidase complex (ABC) method with kit reagents (Vector Laboratories, Netherlands) with diaminobenzidine/H₂O₂ as the chromogenic substrate. After an overnight incubation with the anti-S100A6 antibody (1:3000), the sections were sequentially incubated with appropriate secondary antibodies reagents (Vector Laboratories) conjugated to biotin (1:100), followed by ABC. The peroxidase activity was revealed using diaminobenzidine as chromogen. For immunolabelling with the monoclonal A β antibody (1:1000), rehydrated tissue sections were pre-treated with 100% formic acid for 10 min before incubation with the blocking solution. Controls were performed by omitting the primary antibody.

Table 1
Summary of clinical staging and neuropathological data for AD patients and control patients

Patient #	Sex	Age	Clinical diagnosis	NFT stage	A β IR	S100A6 IR
1	F	43	C	0	–	–
2	M	72	C	0	–	–
3	F	67	C	I	–	–
4	M	69	C	I	–	+
5	F	91	A	V–VI	+	+
6	F	88	A	V–VI	++	++
7	M	84	A	V–VI	+	++
8	M	75	A	V–VI	+++	+++
9	F	76	A	V–VI	++	++

Clinical synopsis ((C) controls; (A) AD patients) and neuropathological staging for NFT (according to Courtois-Coutry et al. [20]. A semi-quantitative estimation for the densities of A β immunoreactive (IR) deposits and of clusters of S100A6-positive astrocytes was performed ((–) absent; (+) weak; (++) moderate; (+++) strong). Note that the patterns of A β and astrocytic S100A6 immunoreactivity are similar.

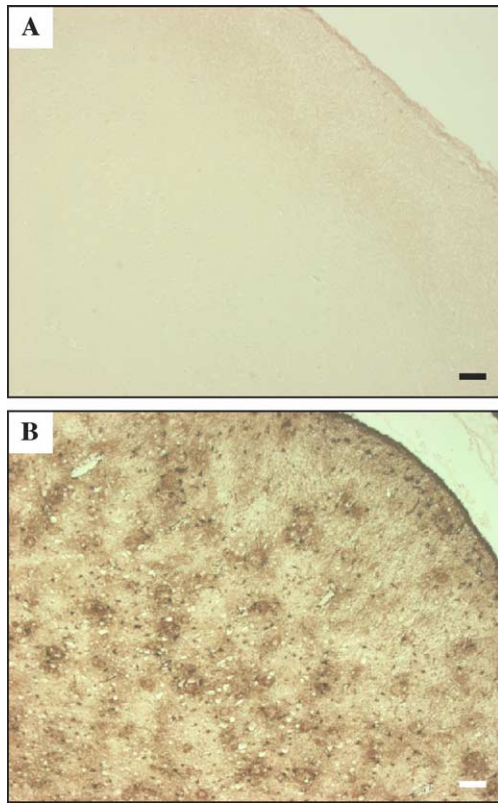


Fig. 1. Coronal section photomicrographs of human temporal neocortex (T5) demonstrating the S100A6-immunohistochemical labelling and its cortical distribution in a neurologically normal subject (panel A) and in one clinically demented case with neuropathologically confirmed AD (panel B), which corresponds to patient #8 in Table 1. Scale bars: 50 μ M.

2.4.2. Double immunohistochemical labelling

For double immunofluorescent labelling, tissue sections were incubated overnight with S100A6 (1:200) and either GFA (1:100), S100B (1:50), tau (1:100) or A β (1:100)

antibodies. Depending on the protocol, the first antibody was detected using either an anti-rabbit, an anti-goat, or an anti-mouse antibody conjugated to Alexa 488 (Molecular Probes) and the second antibody was detected using either an anti-goat, an anti-rabbit or an anti-mouse antibody conjugated to biotin, followed by streptavidin conjugated to Alexa 594 (Molecular Probes).

Tissues were then washed three times with TBS for 10 min, rinsed with water, and mounted with a Gelvatol solution containing 100 mg/ml Dabco reagent. Digital images of the double immunolabelled tissue sections were acquired using an Axiocam HR (Zeiss, Oberkochen, Germany) digital camera.

Double immunolabelling with chromogenic substrates were also performed with the S100A6 polyclonal antibody and the mouse monoclonal antibody to A β . After incubation with the primary antibodies, tissue sections were incubated with a biotin-conjugated horse anti-mouse antibody (Vector Laboratories) and a goat anti-rabbit antibody (Nordic) and then sequentially with streptavidin conjugated to alkaline phosphatase (Boehringer-Mannheim) and with a rabbit peroxidase–antiperoxidase (PAP) complex (Nordic). They were finally incubated with diaminobenzidine (Sigma) as a chromogen to visualize peroxidase activity (brown) and a substrate purchased from Vector to visualize alkaline phosphatase activity (purple).

3. Results

3.1. S100A6 immunoreactivity is weak or absent in the control human temporal cortex

S100A6 distribution within normal temporal cortex level (T5) is depicted in Fig. 1A. Only a weak S100A6

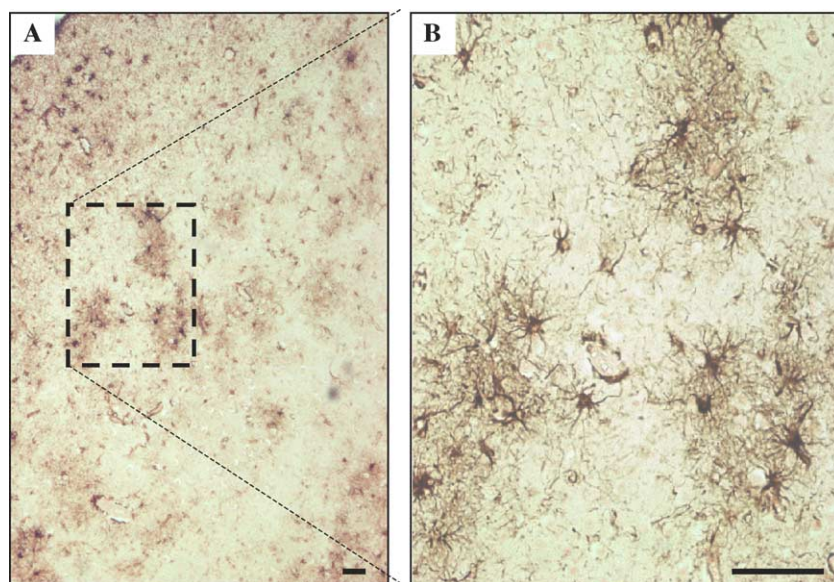


Fig. 2. Panel A, representative images of astrocytic S100A6 immunoreactivity in the neocortex of an AD patient. The area indicated by a dashed box was enlarged and is shown in panel B demonstrating clusters of S100A6-labelled astrocytes associated to senile plaques. Scale bars: 50 μ M.

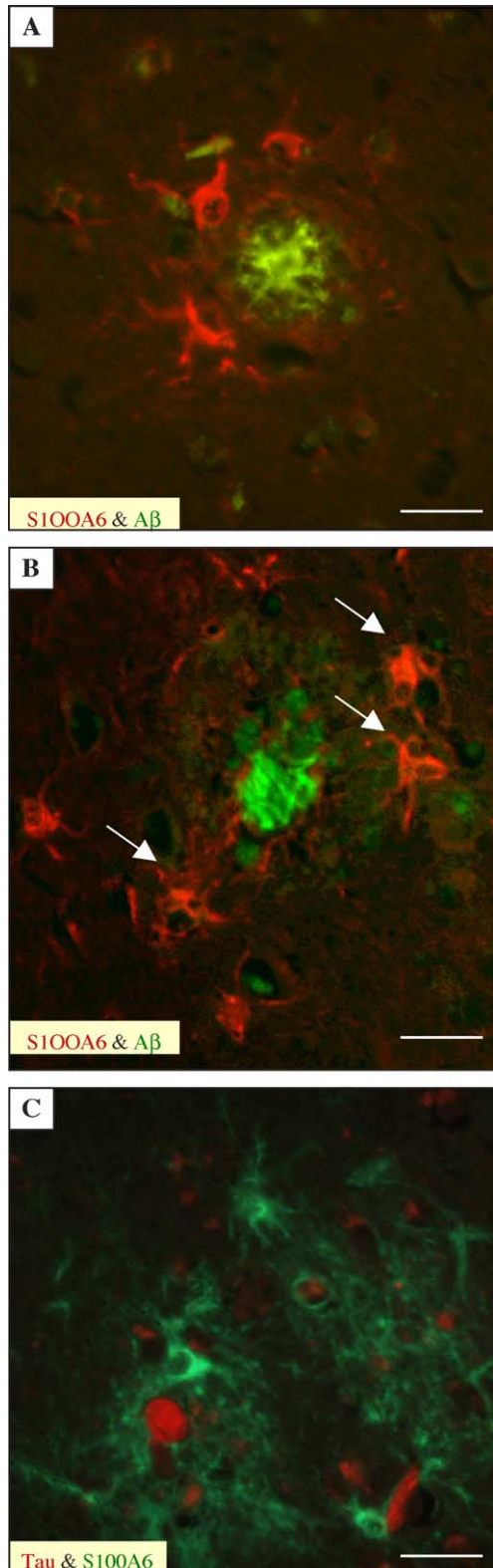


Fig. 3. Panels A and B, double immunolabelling in the temporal cortex of patient with AD demonstrating mature senile plaque deposit (green) surrounded by S100A6 immunoreactive astrocytes (red) located at the periphery of amyloid plaque. Panel C, double immunolabelling in the temporal cortex of patient with AD demonstrating S100A6 labelled astrocytes (green) around an unlabelled amyloid central core in a senile plaque. Adjacent tau-positive dystrophic neurites (red) are also observed. Scale bars: 15 μ M.

immunoreactivity was barely observed at the level of the glia limitans in grey matter. An occasional immunoreactivity (IR) was detected around small vessels, probably at the level of the astrocyte end feet on brain capillaries (data not shown).

3.2. Clusters of S100A6 immunoreactivity in temporal neocortex of AD patients

In contrast to control subjects, conspicuous clusters of S100A6-positive astrocytes were present within the neocortex of AD (Fig. 1B). The number of S100A6 clusters could be correlated to the density of A β deposits associated to senile plaques. Panel B corresponding to patient #8 (see Table 1), exhibits high density of both clusters of S100A6-positive astrocytes and A β deposits. Clustering of S100A6-positive astrocytes throughout the neocortex of AD patient is shown in Fig. 2A and B. The more intensively S100A6-

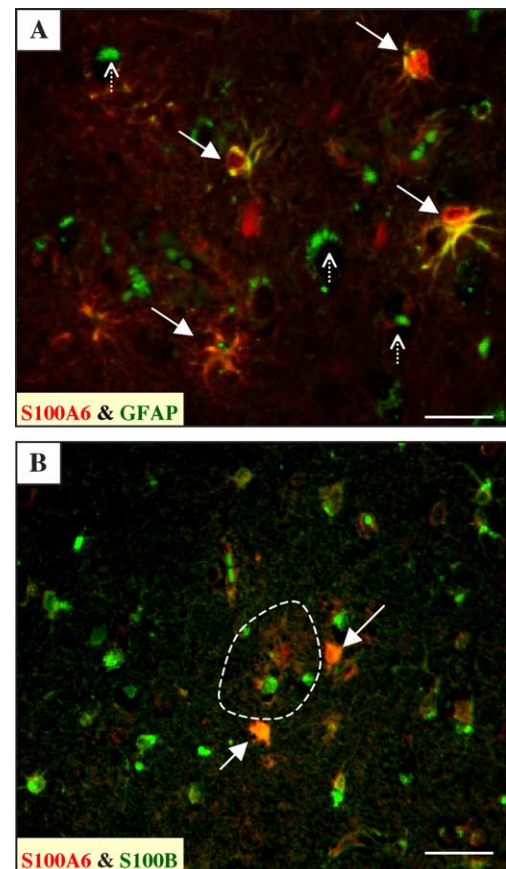


Fig. 4. Panel A, double immunolabelling in the temporal cortex of patient with AD demonstrating S100A6 (red)- and GFAP (green)-positive labelled astrocytes detected in the senile plaques. Co-localisation of both S100A6 and GFAP appears in yellow, indicating that S100A6-positive astrocytes detected in the senile plaques were also GFAP-positive. Dashed arrows in panel A correspond to autofluorescence of lipofuscin granules. Panel B, double immunolabelling in the temporal cortex of patient with AD demonstrating S100A6 (red)- and S100B (green)-positive labelled astrocytes. The reactive astrocytes expressing S100B were found in AD temporal neocortex scattered in the entire grey matter (green), whereas S100A6-positive astrocytes (arrows) were only detected at the level of the senile plaques (dashed circle). Scale bar: 70 μ m.

positive astrocytes were associated to dense core A β deposits. These dense core A β deposits belong to classical plaques with neuritic elements. Many S100A6-positive astrocytes were also observed within the white matter in AD cases, but not in control subjects. On the contrary to the grey matter, these S100A6-positive astrocytes were homogeneously distributed within the white matter (data not shown). In AD temporal neocortex, S100A6 expression both in number of labelled astrocytes and in IR intensity appeared also to be correlated to the NFT staging. The pathological staging according to Braak and Braak [20] as well as a semi-quantitative estimation of the S100A6 IR is summarized in Table 1.

3.3. S100A6 immunoreactivity is associated to a subset of GFAP-positive astrocytes in the AD temporal cortex

To ascertain the preferential association of S100A6-positive astrocytes with the neuropathological lesions of AD, we carried out double immunohistochemical staining experiments on the same tissue sections with S100A6 antibody and either anti-A β (Fig. 3A–B) or anti-tau (Fig. 3C), GFAP (Fig. 4A) and S100B (Fig. 4B) antibodies. Strongly S100A6-IR cells located around the A β deposits were encountered in senile plaques (Fig. 3A–B). Astrocytes surrounding mature plaques containing a central amyloid core stained with Congo red were systemically labelled for

S100A6 (data not shown). In Fig. 3C, a double immunolabelling for S100A6 (green) and tau (red) demonstrates both the S100A6-positive cells and the dystrophic neurites (as well as the neuropil threads) surrounding an amyloid core in a senile plaque. On the contrary to A β deposits, no preferential association was observed between S100A6 immunoreactive cells and tau-positive NFT in neurons (data not shown). Fig. 4A shows that S100A6-positive astrocytes detected in the senile plaques were also GFAP-positive (yellow). Co-localisation was restricted to astrocyte cytoplasm, whereas the nuclei were only S100A6-positive. Dashed arrows in Fig. 4A correspond to autofluorescence of lipofuscin granules.

3.4. S100A6 immunoreactivity is associated with a subset of S100B-positive astrocytes in the AD temporal cortex

In the grey matter of the temporal neocortex of AD, S100B astrocytic expression was clearly up-regulated, both in number of positive astrocytes and in labelling intensity (Fig. 4B). These reactive astrocytes expressing S100B were found in AD temporal neocortex not solely at the level of the A β amyloid deposits but also in astrocytes scattered in the entire grey matter of the neocortex, in areas devoted to senile plaques (Fig. 4B). Thus, on the contrary to the S100A6 phenotype (Fig. 3A and B), the distribution for S100B appears more diffuse in the grey matter.

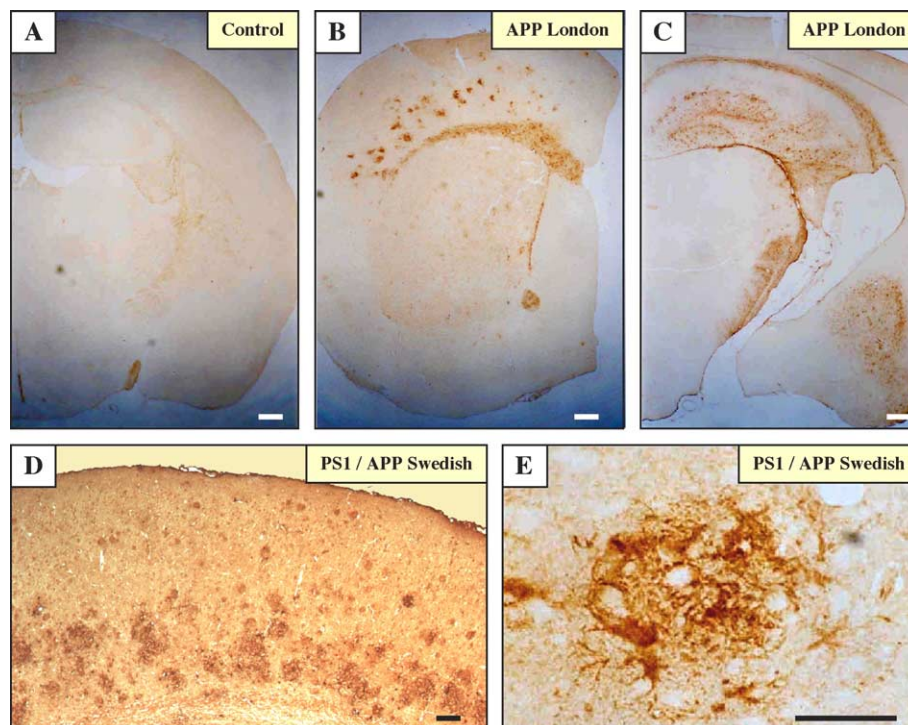


Fig. 5. S100A6 immunolabelling brain from APP London transgenic mice ([V717I] mutation) (panels A, B and C), and in brain from double transgenic mice expressing both a mutant of PS1 protein [M146L] and a mutant of APP-751 protein bearing the Swedish mutations [K670N, M671L] (panels D and E). Astrocytic overexpression of S100A6 was essentially observed in the neocortex and appears to surround the A β amyloid deposits (A–D). The hippocampal formation as well as the amygdaloid nuclei also showed reactive astrocytes (panel C). The white matter (corpus callosum and internal capsule) showed S100A6-positive reactive astrocytes in the transgenic mice with A β deposits (panels B and C) but not in the control mouse (panel A). Scale bars: 25 μ m (B–D).

3.5. *S100A6 immunoreactivity is detected in astrocytes associated to amyloid deposits in transgenic mice*

Transgenic mice expressing one or several human genes involved in AD have proven to be valuable models for the study of the disease [21–23]. Single APP/London and double PS1/APP transgenic mice with neuronal overexpression of the clinical mutants of APP or PS1, develop one of the hallmark of Alzheimer's disease, namely the amyloid plaques in hippocampus and cortex. In these models, it was observed, as in brain from Alzheimer patients, that S100A6 protein immunoreactivity was specifically located within astrocytes associated to the amyloid plaques in the two different transgenic mouse models of AD (Fig. 5B–E). A clear astrocytic labelling for S100A6 was also observed in the hippocampal formation and at the level of amygdaloid nuclei (Fig. 5C). The astrocytes in white matter (internal capsule and corpus callosum) were labelled for S100A6 only in the transgenic mice (Fig. 5B–C) but not in the control mice (Fig. 5A).

4. Discussion

Astrocytic S100A6 overexpression has been associated with damaged axons of motoneurons from human ALS and mice models [11,12]. This enticed us to examine if in another neurodegenerative disease and its relevant mice transgenic models, this specific phenotype could also arise and thereby yield additional evidence and information for a mechanistic-oriented approach. Indeed, we show here a strong S100A6 expression in a subset of reactive astrocytes in AD brain at the level of the senile plaques, one of the pathological hallmarks of this disease. Reactive astrogliosis is a well-known pathological feature of AD, both diffuse and in close association with senile plaques [24,25]. Identification of this subset of S100A6-positive astrocytes is in agreement with the notion that astrocytes constitute a heterogeneous population exhibiting different molecular phenotypes under baseline conditions, after activation [26], and in reactive gliosis [27]. Interestingly, the expression of another member of the S100 family of proteins, S100B, is also up-regulated in a specific subset of astrocytes in the central nervous system with various clinical conditions such as brain trauma [28], inflammatory diseases, ischemia [29], and also during pathological ageing, i.e. AD and Down's syndrome [6,30]. Astrocyte-derived S100B was shown to be the prominent isoform of S100 protein which is abundantly expressed in glia in both white and grey matter, and in some groups of neurons [31]. On the contrary, S100A6 mapping in the rat brain [10] identified immunoreactive cells only in a few regions such as neurons in the limbic system and subpopulation of astrocytes in the white matter, whereas in the grey matter, except the limitans glia, no S100A6-labelled cells were observed. In AD, as we report here for S100A6, it has been shown that activated

astrocytes markedly overexpress the S100B protein [32], especially at the level of the neuritic plaques [33]. S100B is produced mainly by astrocytes and could exert paracrine and autocrine effects on neurons and glia [34] with a dual concentration-dependent behaviour. At nanomolar concentrations, S100B is considered to act as a neurite extension factor and its expression seems to be up-regulated during the development of neuritic plaques in AD [33]. In contrast, micromolar levels of extracellular S100B *in vitro* stimulate the expression of proinflammatory cytokines and induce apoptosis [35]. The observation that S100B and S100A6 form heterodimers and that these heterodimers are implicated in pathological signal transduction in melanoma [36] might be extended to S100B and S100A6 astrocyte overexpression in AD, where they could exert some similar functions. In this study, S100A6 immunoreactivity was systematically prominent in astrocytes associated with classical senile plaques with a dense amyloid core, and to a lesser extent, was also seen in diffuse plaques, considered as an earlier stage. This raises the question of whether it is the highly aggregated form of A β , rather than the non-fibrillary form of A β present in diffuse deposits, that might lead to increased S100A6 expression in reactive astrocytes or if S100A6 might rather contribute to the aggregation of A β . In favour of the first hypothesis, A β has been shown to activate cultured astrocytes, inducing expression of various mediators, e.g. interleukin-1 and nitric oxide release [37] and IL-1 has also been previously shown to induce S100B [35]. In addition, amyloid deposits in senile plaques contain additional (non-A β) components, e.g. α 1-antichymotrypsin [38], which might also play a role in astrocyte activation. Furthermore, the activated microglial cells associated with classical senile plaques might be expected to release a host of biologically active inflammatory molecules [39], potentially leading also to astrocyte activation and increased S100A6 expression. In a previous work, we identified in AD a population of reactive astrocytes overexpressing the tumour suppressor protein APC, product of the adenomatous polyposis coli gene [40]. These APC-positive astrocytes were also preferentially associated with amyloid deposits in classical senile plaques. This suggests that the population of astrocytes associated with dense amyloid deposits in senile plaques exhibit a specific pattern of activation, associated with expression of several molecular markers including S100A6, S100B and APC. Therefore, it would be interesting to look in ALS patients and see if APC is also co-up-regulated with S100A6. This would discriminate between a broad unspecific activation and a more specific mechanism. The clear correlation between the dense neuritic plaques and immunoreactive S100A6 astrocytes additionally illustrates that in two neurodegenerative diseases (ALS and AD) and in associated animal models, a specific subpopulation of astrocytes overexpress a Ca²⁺/Zn²⁺ ion binding protein. Considering the Ca²⁺ and Zn²⁺ binding properties of S100A6, there are further indications that S100A6 (that has an even higher affinity for zinc ions than

for calcium ions) might play a role in the regulation of the homeostasis of these cations in AD. First, aggregated A β amyloid is known to increase intraneuronal calcium ions by various mechanisms [41–43]. Secondly zinc ions are enriched in the neocortical areas, and even further concentrated in the amyloid deposits in AD [44]. Thirdly, in the APP/London mice, S100A6 up-regulation was observed in the amygdala and hippocampus area, both regions impaired in AD and known to be densely innervated by zinc-containing neurons. In conclusion, the observation of a specific astrocytic expression of S100A6 in the brain of AD patients and in two independent AD mouse models, not only validate these models, but justify additional studies to clarify the functional relevance of this astrocytic S100A6 overexpression. An eventual trophic or detrimental role for S100A6 cannot be excluded and further investigations are needed to verify a possible buffering role of S100A6 for biometals such as zinc. Finally, as S100A6 was first discovered as a protein expressed during fibroblast proliferation [45], it would be of interest to investigate if the observed S100A6-positive astrocytes are indeed entering into cell division cycle.

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